Modulation of IL-10, IL-12, and IFN-γ in the Epidermis of Hairless Mice by UVA (320–400 nm) and UVB (280–320 nm) Radiation

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We have observed recently that the suppression of contact hypersensitivity (CHS) induced in mice by UVB irradiation may be prevented by suberythemal exposure to UVA radiation. Because the UVB-immunosuppressed state is associated with an upregulation of the Th2-associated cytokines IL-10 and IL-4, and a deficiency in Th1-associated IL-2, IL-12, and IFN-γ, and because UVA photoimmunoprotection appeared to be IFN-γ- dependent, we tested the hypothesis that UVA immunoprotection results from an ability to prevent the UVB-induced cytokine disarray. This study describes changes in epidermal IL-10, IL-12 and IFN-γ for 5 d following irradiation of hairless mice with the CHS-modulating doses of UVB, UVA, or UVA + UVB, using immunohistochemical detection in paraffin embedded skin sections, followed by image analysis quantitation. We found that UVB, but not UVA exposure, caused an increase in epidermal IL-10 expression, peaking at 3 d. UVA irradiation, but not UVB, resulted in increased epidermal IL-12 expression, peaking at 3 d, and increased epidermal IFN-γ expression peaking earlier at 1 d. Irradiation with UVA + UVB abrogated the UVB-enhanced expression of IL-10, and caused small but significant increases in IL-12 and IFN-γ at 3 d and 1 d, respectively. These findings suggest that UVA photoimmunoprotection is mediated via prevention of IL-10 release, and thus the maintenance of the Th1/Th2 balance, probably by upregulation of IL-12 and IFN-γ, which are known to antagonize IL-10 in numerous models. The time course suggests that IFN-γ responds initially to UVA radiation, and may stimulate the increased expression of IL-12. ***Key words: cytokine/image analysis/immunohistochemistry. J Invest Dermatol 113:1059–1064, 1999***

The suppression of cell-mediated immunity resulting from exposure to UVB (280–320 nm) radiation appears to be correlated with an imbalance in the cutaneous cytokine array, such that T helper (Th)2 associated cytokines predominate at the expense of Th1 associated cytokines (Ullrich, 1996). The anti-inflammatory Th2 cytokine IL-10 has been identified as a major mediator of photoimmunosuppression (Rivas and Ullrich, 1992), and its expression is increased following UVB irradiation of cultured keratinocytes (Grewe *et al*, 1995), in monocyte/macrophage or T lymphocyte populations and in serum from UVB-irradiated mice and humans (Kang *et al*, 1998; Shreedhar *et al*, 1998; Ullrich *et al*, 1998; Yoshida *et al*, 1998). On the other hand, expression of the Th1 cytokines IL-12 and IFN-γ is decreased following UVB irradiation (Khan *et al*, 1993; Teunissen *et al*, 1993; Simon *et al*, 1994; Kremer *et al*, 1996), and photoimmunosuppression in mice has been inhibited by treatment with recombinant IL-12 (Muller *et al*, 1995; Schmitt *et al*, 1995; Schwarz *et al*, 1996).

In contrast to these UVB-induced effects, the immunologic consequences of exposure to UVA (320–400 nm) radiation are less well understood. We have recently observed that suberythemal UVA exposure of hairless mice is itself immunologically innocuous, but induces a state of resistance to the systemic suppression of CHS by either UVB radiation or exogenous *cis*-urocanic acid, a natural epidermal UVB-photoproduct that acts as a mediator of the photoimmunosuppression (Reeve and Ley, 1997; Reeve *et al*, 1998). In seeking the mechanism of such photoimmunoprotection by UVA radiation, we have hypothesized that UVA radiation might prevent the characteristic cytokine imbalance produced in the skin by UVB radiation. In studies using IFN-γ gene knockout mice, we subsequently found that the UVA effect was dependent on IFN-γ (Reeve *et al*, 1999), supporting the hypothesis.

The Th1 cytokines IFN-γ and IL-12 are closely interrelated. IL-12, which protects from photoimmunosuppression in mice, apparently by blocking IL-10 secretion in UVB-irradiated keratinocytes,[1] is known to stimulate IFN-γ expression (Trinchieri, 1993). In addition, a positive feedback effect has been described by which IFN-γ enhances IL-12 production, and these cytokines block IL-10 formation by macrophages and T cells (Chomarat *et al*, 1993; Marshall *et al*, 1995). The interplay between the Th1 immunopotentiating or Th2 immunosuppressive cytokines within the UV-irradiated cutaneous environment is thus complex. In this immunohistochemical study in the hairless mouse,

Manuscript received April 16, 1999; revised July 22, 1999; accepted for publication August 26, 1999.

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Abbreviations: CHS, contact hypersensitivity; MED, minimal erythemal dose; Th, T helper.

[1] Schmitt DA, Ullrich SE: Interleukin (IL)-12 overcomes UVB-induced immune suppression by preventing the production of IL-10 by keratinocytes. *Photochem Photobiol* 65:84S, 1997 (abstr.)

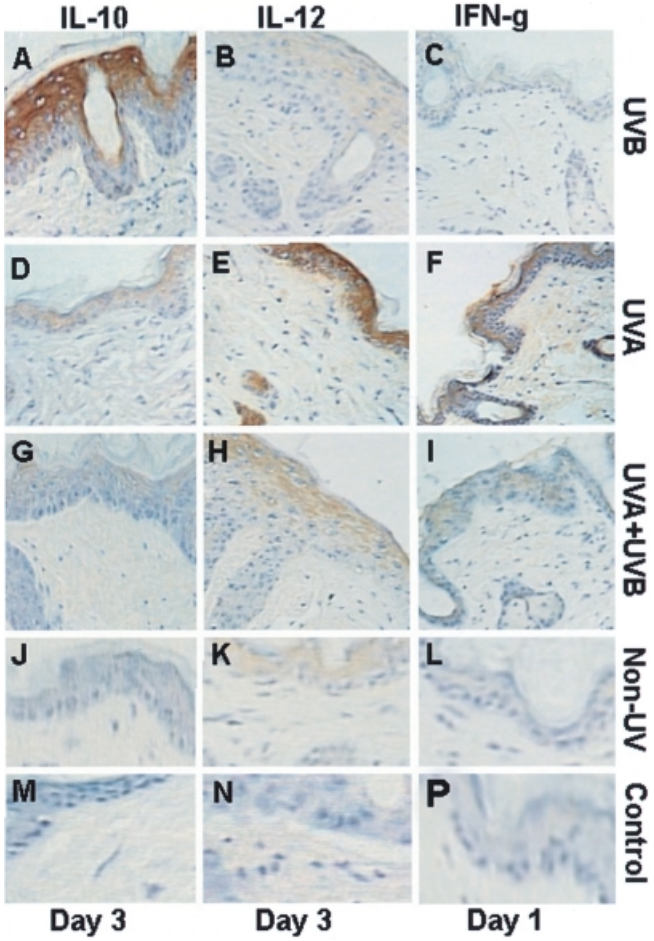

Figure 1. Immunohistochemical demonstration of epidermal cytokine expression at critical time points postirradiation. Peak expression at 3 d postirradiation of IL-10 (induced by UVB, but not by UVA or UVA + UVB) and IL-12 (induced by UVA, but not by UVB), and of IFN-γ (induced by UVA but not by UVB) at 1 d postirradiation. Constitutive cytokine staining is also shown (Non-UV), along with the isotype control staining (Control).

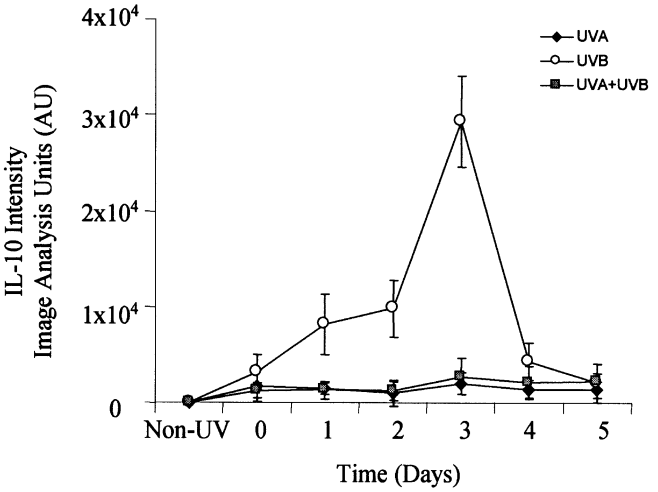

Figure 2. Post-irradiation time course of the expression of IL-10 quantitated by image analysis. Skin sections were analyzed before (Non-UV), immediately following (0 d), and at daily intervals for 5 d following exposure to UVA, UVB, or UVA + UVB radiation. Points are the mean ± SD of at least 30 fields from each section, taken from three mice per treatment.

we report the progressive alterations in three critical cytokines (IL-12, IFN-γ, and IL-10) as a time course following exposure of mice to the immune modifying doses of UVB or UVA radiation, and the combination treatment of UVA followed by UVB radiation, with the aim of correlating the immunopositive cytokine presence with the established alteration to immune function.

MATERIALS AND METHODS

Animals Female inbred albino Skh:HR-1 hairless mice were provided from the Veterinary Pathology breeding colony. The mice were aged 12–14 wk and were fed with stock laboratory mouse cubes (Norco Stockfeeds, Lismore, Australia) and tap water *ad libitum*. Groups of 3–4 mice were kept in wire-topped plastic boxes on vermiculite bedding and maintained at 25°C under gold lighting (GEC F40GO tubes), which does not emit UVB radiation, on a 12 h on/off cycle. The study was conducted according to the University of Sydney Animal Ethic Committee guidelines, conforming to the N.S.W. Animal Welfare Act.

UV irradiation The radiation sources were made up of 120 cm fluorescent tubes as described previously (Reeve *et al*, 1998), and irradiance was monitored using an International Light IL1500 radiometer with appropriately calibrated detectors for the UVA and UVB wavebands. The UVB radiation was provided by a single unfiltered UVB tube (Westinghouse FS40 type: Oliphant FL40SE, Oliphant-UV, Adelaide, S.A.) emitting 2.5×10^{-4} W UVA per cm^2 and 4.1×10^{-4} W UVB per cm^2. The UVA source was a planar bank of seven UVA tubes (Hitachi F40/T 10BL) filtered through a sheet of 6 mm window glass, providing 2.7×10^{-3} W UVA per cm^2 and 2.3×10^{-8} W UVB per cm^2.

Mice were irradiated with a single exposure of either UVA or UVB, or were irradiated with UVA followed immediately by UVB. Thus they were exposed on the dorsum, unrestrained in their boxes with the wire tops removed, to UVA radiation (4 h exposure; 387.4 kJ UVA per m^2), which is suberythemal, approximately equal to the UVA content of 5–10 times the minimal erythemal dose (MED) of summer sunlight in humans (Reeve *et al*, 1998), and contains a negligible UVB content (0.0033 kJ UVB per m^2). Alternatively, they received a UVB exposure approximately equal to 3 'MED" (3.4 kJ UVA per m^2, 5.54 kJ UVB per m^2), the "MED" having been previously defined in this mouse by its skinfold thickness response, which is primarily due to edema, at 24 h postirradiation (Reeve *et al*, 1998). Groups of mice were also irradiated with the 4 h UVA exposure followed immediately with the UVB exposure. Temperature was controlled with an electric fan, and the boxes of mice were gently shaken regularly to prevent shielding by sleeping cage mates, especially during the longer exposure times with UVA.

Cytokine detection by immunohistochemistry Three or four mice per treatment were euthanased before and at various time points (immediately, 1, 2, 3, 4, and 5 d) following irradiation, and dorsal skin samples excised, fixed in Histochoice (Amresco, Solon, OH), processed, and paraffin-embedded. Sections were cut at 5 μm onto polylysine-coated slides. These were then de-waxed and rehydrated through graded ethanol solutions to phosphate-buffered saline (PBS; 0.15 M NaCl with 10 mM potassium phosphate, pH 7.3). Endogenous peroxidase activity was quenched by incubation in 3% (vol/vol) hydrogen peroxide in methanol (Sigma Aldrich Pty, Castle Hill, N.S.W.) for 30 min. The sections were then blocked with normal nonimmune goat serum (CSL Biosciences, North Ryde, N.S.W.) (1:100 dilution) at room temperature for 30 min to remove nonspecific binding sites for the second antibody. The tissues were then washed twice in PBS and incubated with goat anti-IL-12 (1:200), anti-IL-10 (1:200), or anti-IFN-γ (1:100) antibodies (R&D Systems Pty, Gymea, N.S.W.) diluted in DMEM (Sigma), for 2 h at room temperature. Anti-IFN-γ, anti-IL-10, and anti-IL-12 antibodies are specific for the corresponding cytokine only, and there is no cross-reaction with cytokine receptor, according to the manufacturer's information. The anti-IL-12 antibody recognizes both p35 and p40 antigens, according to the manufacturer's information. Irrelevant antibody of the same isotype was used as control for nonspecific antibody binding in each case. After washing with PBS, the slides were incubated at room temperature for 1 h with biotinylated goat antibody made from horse (1:100) (Vector), followed by incubation of slides in avidin-horseradish peroxidase conjugate (1:100) (BioRad, Regents Park, N.S.W.) for 30 min. Finally, the slides were developed in 3,3′-diaminobenzidine (Vector DAB kit) for 5 min at room temperature and mounted in DPX. The stained skin sections were examined using the Leica Q500 MC image processing and analysis system

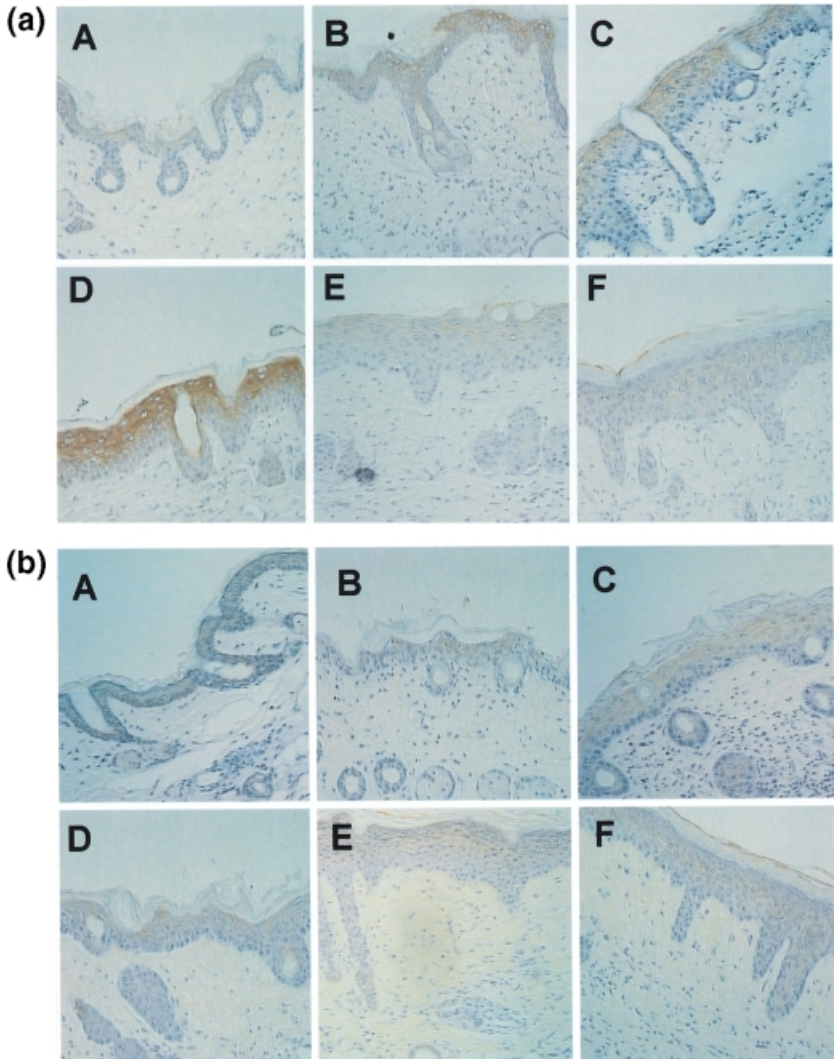

Figure 3. Time course of immunopositive IL-10 expression in epidermis following UVB and UVA + UVB irradiation. (*a*) UVB; (*b*) UVA + UVB. A, immediately postirradiation; B, C, D, E, F at 1 d, 2 d, 3 d, 4 d, and 5 d postirradiation, respectively.

(Cole *et al*, 1999). Positive targets in the skin epidermal layer were identified and designated on the displayed image. This definition of positive was used for analysis for all subsequent samples. The image analysis results (arbitrary image analysis units) are represented as the average of positive staining area per skin section, after correction for increases in epidermal thickness resulting from UVB irradiation in particular. A minimum of 30 randomly selected fields per section from each mouse were analyzed. As there was no significant variation between the individual mice within the group, the mean values ± SD were calculated (90+ fields) for each treatment group. Differences between treatments were examined for significance with a two-tailed unpaired Student's t test, comparing either sequential time points within the same treatment or separate treatments at the same time point. The computerized images of the stained sections were also recorded photographically with the Adobe PhotoShop program.

RESULTS

Expression of IL-10 The selected cytokines were detected under our staining conditions only in the epidermal cutaneous compartment. Staining was diffuse and indicated the extracellular diffusion of the cytokines.

There was no detectable IL-10 in the normal epidermis (**Fig 1*J***) compared with the isotype control stained section (**Fig 1*M***). Regardless of the radiation source, exposure resulted in detectable IL-10 immediately (day 0) at similar concentrations (**Fig 2**); however, at 1 d post-UVB irradiation there was a highly significant ($p < 0.00001$) increase in IL-10 expression, which continued to rise until peak expression at day 3 (approximately 12-fold increase from day 0), after which it decreased, remaining detectable at day 4, and returning to a nonsignificant level at day 5. The time course of this IL-10 response to UVB irradiation is shown histologically in **Fig 3**(***a***), and by image analysis in **Fig 2**.

On the other hand, UVA irradiation did not cause such an increase in IL-10 expression, as shown histologically at day 3 (**Fig 1*D***), and although IL-10 was detectable for 5 d post-UVA irradiation, the concentration was consistently low (**Fig 2**). The combination of UVA + UVB irradiation (**Fig 3*b***) did not significantly change the low IL-10 response to UVA irradiation alone ($p > 0.05$; **Fig 1*G***). In striking contrast to UVB irradiation, however, UVA + UVB treatment completely abrogated the sequence of enhanced IL-10 production between day 1 and day 4, which culminated in the marked UVB-induced peak of IL-10 production at day 3, shown histologically in **Fig 3**(***b***), and by image analysis in **Fig 2**.

Expression of IL-12 In contrast with IL-10, constitutive expression of IL-12 was detected in the normal epidermis (**Fig 1*K***) compared with the isotype control (**Fig 1*N***). Irradiation with UVB resulted in a significant ($p = 0.002$) decrease in the normal IL-12 expression at day 1. The low expression was maintained until day 4, and reduced even further almost to an undetectable level at day 5 (**Fig 4**).

In contrast, UVA irradiation resulted in a significant ($p = 0.007$) increase in IL-12 staining at day 1, which continued to rise until a peak (approximately 6-fold increase) at day 3, shown histologically

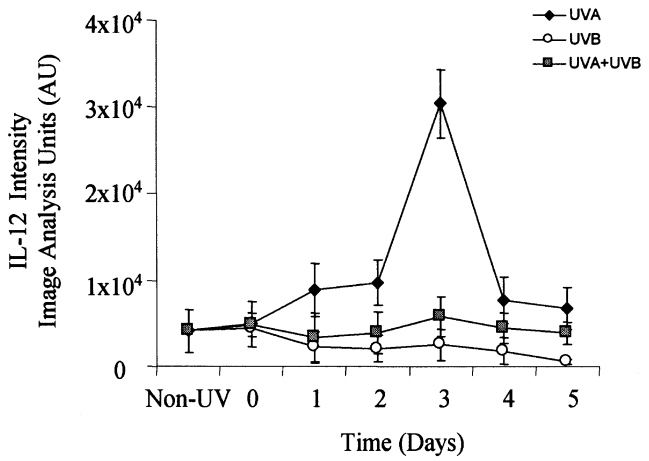

Figure 4. Post-irradiation time course of the expression of IL-12 quantitated by image analysis. Skin sections were analyzed before (Non-UV), immediately following (0 d), and at daily intervals for 5 d following exposure to UVA, UVB, or UVA + UVB radiation. Points are the mean ± SD of at least 30 fields from each section, taken from three mice per treatment.

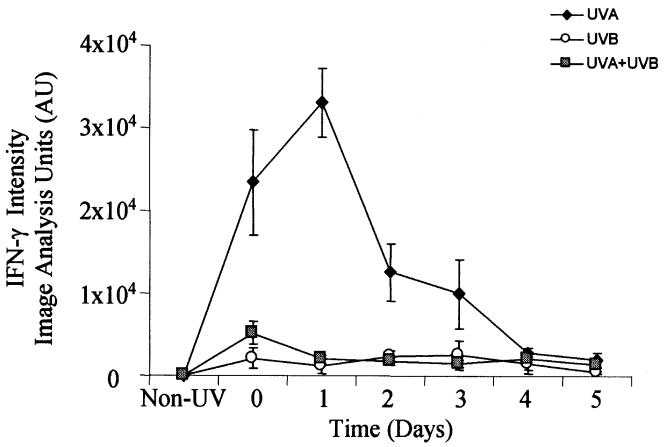

Figure 5. Post-irradiation time course of the expression of IFN-γ quantitated by image analysis. Skin sections were analyzed before (Non-UV), immediately following (0 d), and at daily intervals for 5 d following exposure to UVA, UVB, or UVA + UVB radiation. Points are the mean ± SD of at least 30 fields from each section, taken from three mice per treatment.

in **Fig 1**(***E***), and by image analysis in **Fig 4**, and then declined by day 5. The combination of UVA + UVB irradiation did not cause such a strong increase in IL-12 expression; however, although the level of staining remained markedly less than following UVA irradiation, there was a slight but significant ($p = 0.03$ at day 1) increase in IL-12 expression following UVA + UVB compared with the response to UVB radiation, and this became more significant at day 3 ($p < 0.00001$; **Fig 1***H*) and remained higher until day 5 (**Fig 4**).

Expression of IFN-γ There was no detectable IFN-γ in the normal epidermis (**Fig 1***L*) compared with the isotype control (**Fig 1***P*). Irradiation with UVB resulted in a small but significant ($p < 0.05$) presence of IFN-γ immediately (day 0), which remained detectable until day 5 (**Fig 5**). In contrast, UVA irradiation resulted in an immediate strong increase in IFN-γ expression at day 0, which peaked at day 1, as shown histologically in **Fig 1**(***F***) and by image analysis in **Fig 5**, then subsided slowly until day 5. Combination exposure to UVA + UVB also slightly but highly significantly ($p < 0.0001$) increased IFN-γ staining immediately (day 0), but this expression decreased at day 1 (**Figs 1***I*, **5**), although remaining detectable until day 5. Nevertheless, at the early day 0 time point, the small peak in IFN-γ production after UVA + UVB irradiation was significantly ($p < 0.0001$) greater than the IFN-γ concentration following UVB irradiation.

DISCUSSION

This study has demonstrated that irradiation of hairless mouse skin with a single exposure of UVB radiation sufficient to cause approximately 50% suppression of systemic CHS (Reeve *et al*, 1998), causes a wave of increased production of the Th2 cytokine IL-10 in the epidermis, peaking at 3 d. Irradiation with a single exposure of UVA radiation, which we reported previously to have no effect on systemic CHS induction, did not produce significant levels of IL-10. The most striking finding was that UVA exposure preceding the UVB exposure prevented the expected UVB stimulation of IL-10 production and only minimal levels of IL-10 could be detected in the epidermis for the next 5 d. This combination of UVA + UVB radiation was previously found to prevent the suppression of systemic CHS caused by UVB radiation alone. Thus our observations are consistent with the increase in epidermal IL-10 playing a major role in establishing the photoimmunosuppressed state, in agreement with the observations of others (Rivas and Ullrich, 1992). The study also indicates that the protection from UVB-induced immunosuppression by UVA exposure is achieved by the prevention of this IL-10 release.

Furthermore, UVA irradiation resulted in markedly increased expression of both IL-12 and IFN-γ, whereas these Th1 cytokines were detectable only at very low levels in the epidermis following UVB irradiation. These findings are in accord with a recent study in cultured human keratinocytes, in which UVA irradiation *in vitro* induced IL-12 mRNA and protein, but not IL-10 (Kondo and Jimbow, 1998), and together the studies support a UVA-dependent predominance of Th1 cytokines. Keratinocytes have not previously been demonstrated to produce IFN-γ; however, a probable source of this cytokine may be the epidermal T cells, which have been shown recently to produce IFN-γ very rapidly in normal human skin upon activation (Hassan-Zahraee *et al*, 1988), and in psoriatic lesional skin (Szabo *et al*, 1998).

As the balance between IFN-γ and IL-12 on the one hand, and IL-10 on the other, has been shown by others to determine CHS responses in mice (Schmitt *et al*, 1995; Xu *et al*, 1996), and in this study, the peak of UVB-induced IL-10 expression, and of UVA-induced IL-12 expression, have coincided at day 3 postirradiation, we suggest that when UVA and UVB radiation are combined (UVA + UVB), the essential interaction of the cytokines involves the suppression of IL-10 release by IL-12. It is not clear at present how this occurs, but the observed increase in IL-12 staining at day 3 following UVA + UVB, at a level significantly greater than the response to UVB, but substantially less than the peak response to UVA radiation alone, may have functional relevance. The relationship between the relative cytokine levels in the epidermis and the consequent alterations in immune function is unknown, and there is evidence that epidermal cytokine levels may be less indicative of the systemic modulation of immune function than levels in the serum (Shreedhar *et al*, 1998), which should be monitored in parallel in future studies. An explanation for the rapid elimination of most of the epidermal IFN-γ appearing immediately after UVA exposure when this is followed by UVB might be loss of this cytokine to the serum. Examination of the cytokine patterns if UVB irradiation precedes UVA exposure might clarify some observations, although the order of irradiation made no difference to the effect on contact hypersensitivity (Reeve *et al*, 1998); UVA concomitant with UVB has also been found to be immunoprotective (Reeve *et al*, 1994). It is also likely that, in addition to the three cytokines we have studied, other cytokines like IL-4, IL-6, known to be modulated by UV radiation or costimulatory molecules like CD-86, may be involved (Marshall *et al*, 1995;

Elghorr and Norval, 1997; Ullrich *et al*, 1998; Nishimura *et al*, 1999).

The relationship between IL-12 and IFN-γ is complex, the function of IL-12 having originally been described as stimulating the upregulation of IFN-γ (Trinchieri, 1993). IL-12 has dominant effects on promoting Th1 development (Manetti *et al*, 1993), and has been found to inhibit the secretion of IL-10 by UVB-irradiated keratinocytes *in vitro*;[1] however, IFN-γ can act in a positive feedback manner by stimulating IL-12 production, also blocking endogenous IL-10 production by macrophages (Chomarat *et al*, 1993), and being itself downregulated by IL-10 (Kondo *et al*, 1994) or upregulated by anti-IL-10 antibody (Arai *et al*, 1995). In addition, the depletion of IFN-γ from monocytes by UVB irradiation has been shown to be restored by recombinant IL-12 (Kremer *et al*, 1996), and Th1 cell development induced by IL-12 could be blocked by anti-IFN-γ antibody (Seder *et al*, 1993). Thus IL-12 and IFN-γ act in a complementary manner, their mutual interregulation making it difficult to separate the specific activities. In a recent study in mice bearing a targeted disruption of the IFN-γ gene (IFN-γ–/–), however, we have observed the failure of the photoimmunoprotective effect of UVA radiation, and its restoration by injected recombinant IFN-γ suggesting that the critical Th1 cytokine for the UVA protective effect is IFN-γ (Reeve *et al*, 1999). This observation is inconsistent with a preliminary report in conventional mice[1] providing evidence that anti-IFN-γ monoclonal antibody had no effect on the ability of IL-12 to overcome UVB immunosuppression; however, the IL-12 status of the IFN-γ–/– mouse has not been examined.

We observed that the elevation of IFN-γ in response to UVA radiation was more rapid and earlier than the IL-12 response, and was already high immediately postirradiation (day 0), with maximum staining at day 1. This is in agreement with the reported presence of mRNA for IFN-γ in normal epidermal T cells, and the rapid translation resulting from their activation at least by mitogen (Hassan-Zahraee *et al*, 1998). It implies that UVA induction of IFN-γ precedes and is involved in the later increase in IL-12 expression, a conclusion that is in agreement with our evidence from the IFN-γ–/– mouse for the essential role of IFN-γ in UVA photoimmunoprotection. The concept that IFN-γ may cause the upregulation of IL-12, either directly or indirectly, has been suggested in past studies of noncutaneous systems in which macrophage IL-12 induction was dependent on both IFN-γ and TNF-α produced by bacterial infection (Flesch *et al*, 1995). In the UVB-irradiated human skin, TNF-α is elevated in blister fluid (Skov *et al*, 1998); and in transgenic mice with a disruption of the TNF receptor, a significant decrease in IL-12 secretion has been described (Zhan and Cheers, 1998). Obviously it would be of additional interest to pursue the changes of cutaneous TNF-α in response to our UV irradiation sources.

In summary, this study has shown by immunohistochemical techniques, that cytokine alterations occurring in the 5 d following exposure to UVA, UVB, or UVA + UVB radiation, may provide a mechanism by which UVA irradiation exerts its photoimmunoprotection against UVB radiation. We have observed that strong upregulation of epidermal IL-10 by UVB exposure correlated with the previously established suppression of CHS, whereas this stimulation of IL-10 expression was prevented if UVA irradiation preceded the UVB exposure. Because the UVA irradiation itself resulted in upregulated IL-12 and IFN-γ expression, the ability of these and possible other Th1 cytokines to inhibit the release of the suppressive IL-10 or possible other Th2 cytokines, may provide the pathway by which the Th1/Th2 cytokine balance is maintained as normal by irradiation with UVA + UVB, and CHS responsivity remains unaffected as a consequence.

We gratefully acknowledge the helpful discussions with Dr. Noriko Nishimura, NIEHS, Tsukuba, Japan, and careful mouse husbandry of Chandrika Abeywardana and John Fisher. This project was supported by the University of Sydney Cancer Research Fund.

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